A New Method for Measuring Viability of Deformed Cells

Takamatsu, Hiroshi
Institute of Advanced Material Study Kyushu University

https://doi.org/10.15017/7906

出版情報：九州大学機能物質科学研究所報告. 13 (1), pp.21-24, 1999-07-08. 九州大学機能物質科学研究所
バージョン：
権利関係：

A New Method for Measuring Viability of Deformed Cells

Hiroshi TAKAMATSU

A new experimental procedure has been developed to measure the viability of isolated cells deformed by two parallel plates which simulate ice crystals under freezing of biological material. The viability of deformed cell is evaluated in situ with trypan blue dye exclusion assay. The change in the viability with the gap size between two plates is obtained for the prostate cancer cell line ND-1 (20 μm in the mean diameter) at about 23 °C. The viability decreases steeply with decreasing the gap size at about 6 μm that is 30% of original cell diameter and about 50% of cells are destroyed. If uniform expansion of cell membrane is assumed, this critical expansion corresponds to the 50% increase in the surface area.

Introduction

The mechanism of freezing injury of cells has been a major interest of cryobiologists. After a number of works on this subject, it has become accepted that the freezing injury occurs due to intracellular ice formation at quick freezing and extracellular ice formation followed by cellular dehydration at slow freezing. The slow freezing injury is possibly caused by (a) chemical damage due to increased concentration of intracellular electrolytes (Lovelock[1], Lovelock[2], Pegg and Diaper[3], Pegg and Diaper[4]), (b) mechanical damage due to volume change of cells (Meryman[5]) and (c) mechanical damage by extracellular ice crystal itself or interaction between packed cells (Nei[6], Nei[7], Mazur et al.[8], Mazur and Rigopoulos[9]). Recently, Ishiguro and Rubinsky[10] observed that during freezing of suspension of red blood cells in the physiological saline without cryoprotective agent the cells were entrapped in the unfrozen solution channels between finger-like crystals. It demonstrates that the mechanical interaction between cells and ice crystal can cause damage. To understand the quantitative effect of this interaction, it is important to know the mechanical properties of cells especially the degree of deformation that causes cell destruction.

Experiments and models for the mechanical properties of biomembranes are reviewed by Evans and Skalak[11] and later works are found in the other books (Bereiter-Hahn et al.[12], Mow et al.[13]). Most of the past research for animal cells have been done with blood cells or sea urchin eggs to make contribution toward understanding of blood rheology or cellular phenomena such as fertilization and cell division. Typical experimental methods include micropipet aspiration, compression between two flat surfaces, and deflection of the surface by a rigid spherical particle. The primary goal of these studies was to obtain stress-strain relation of membranes.

In the present paper, a new method is proposed to determine quantitatively the degree of membrane deformation that causes cell destruction, to understand the possible mechanism of cell damage by ice crystals. A simple model to express the experimental result is also presented. Since this study is a part of work on prostate cryosurgery, the prostate cancer cell is used in the experiment. The prostate cancer is the most common cancer and the second most frequent cause of cancer death in men in the United States, and also the most promising application of cryosurgery (Onik et al.[14]).

Materials and Methods

The experiments were performed with a human primary prostatic adenocarcinoma cell line (ND-1) developed by Narayan and Dahiya[15]. The cell is almost spherical with the mean diameter of 20 μm as shown in Fig. 1. Hiramoto[16] measured the mechanical property of sea urchin egg compressed by two parallel plates using a small glass beam and a glass plate. However, this sophisticated method is not applicable for the present study due to difficulty in determination of cell viability as well as difficulty in handling cells much smaller than the sea urchin egg (95 μm in diameter). In the present experiments, the cells and glass beads suspended in the physiological saline were put on a microslide and compressed by a cover slip. Thereby, the glass beads, which are randomly distributed throughout the solution, behave as spacers with precisely known dimensions. The viability of cell was evaluated with the trypan blue dye exclusion assay. The nonviable cells are visually distinguished from the viable cells because nonviable cells take up the dye, while viable cells exclude the dye.

Received May 25, 1999

The protocol of the experimental method is as follows:

(1) Make glass beads suspension with DPBS (Dulbecco's phosphate buffered saline).

(2) Prepare cell suspension by adding DPBS to centrifuged cells.

(3) Mix 50 μl of cell suspension with 50 μl of glass bead suspension.

(4) Add 100 μl of trypan blue solution (0.4%) to this suspension.

(5) Put appropriate amount of the suspension on a microslide.

(6) Put a cover glass (25 x 25 mm^2) gently on the suspension and compress softly by hand. Remove excessive suspension if necessary to make the gap minimum by capillary force.

(7) Put the microslide on the stage of the microscope and check the area where cells and beads are uniformly distributed.

(8) Observe the area with a 10x objective and record both the bright field and the phase contrast images in the computer using CCD camera.

(9) Move the microslide 1 mm and record the images. Repeat the same operation until enough number of images is obtained.

(10) Repeat operations from (4) to (9) with different type of beads.

All processes were done at the room temperature (about 23 °C). Glass beads (Duke Scientific Corporation) with four different diameters were used: type #1 with 5.1±0.5 μm in the mean diameter and 0.8 μm in the standard deviation; type #2 with 7.8±0.8 μm in the mean diameter and 1.0 μm in the standard deviation; type #3 with 10.4±1.0 μm in the mean diameter and 1.0 μm in the standard deviation; type #4 with 30.2±2.1 μm in the mean diameter and 1.8 μm in the standard deviation. The type #4 was used for the undeformed control and observation with this was performed before and after the experiment to ensure that the viability of control did not decrease during the experimental procedure. Basically two other types of beads were used in a series of experiment, so that an experiment proceeded as, for instance, #4, #3, #1 and #4 in that order. A series of experiment was finished within an hour. Since the glass beads have some scatter in diameter, the gap between the plates depends on the larger beads in the suspension. The nominal gap size h is therefore determined as the sum of the mean diameter and the standard deviation: h = 5.9 μm for #1, h = 8.8 μm for #2, h = 11.4 μm for #3.

The viability of cells was defined by the number of non-stained cells divided by the number of total cells. Counting cells was done after each series of experiment using recorded images. The phase contrast image that is useful for observing non-stained cell was used to count total number of cells, while the number of stained cell was counted with the bright field image. More images were recorded for smaller gap to avoid large difference in the number of counted cells. For a given gap in an experiment more than ten pairs of images were recorded and cell count exceeded a total of 500. The viability was evaluated for each image and normalized by the average viability of the control.

The cells undergo mechanical stresses during centrifuging and mixing by pipet. Attention was paid to avoid difference in these operations among each experiment and the data for the experiments with the viability of control less than 80% was discarded.

Experimental Results

Figure 1 shows the distribution of cell diameter d that is the average value of the maximum and the minimum diameters. This is the result of 542 cells observed with the beads #4 as the spacer. The solid line shows the Gaussian distribution

$$f(d) = \frac{1}{\sigma\sqrt{2\pi}} \exp\left[-(d - d_{med})^2 / 2\sigma^2\right], \quad (1)$$

with d_{med} = 20 μm and σ = 2.0. Since the ratio of the minimum to the maximum diameters was larger than 0.9 for 73% of the measured cells, the cells used in the present study is nearly spherical with the mean diameter of 20 μm.

Figure 2 shows close up views of (a) control cells and (b) the cells for h = 5.9 μm. One large glass bead (#4) and three small ones (#1) are observed in the photos (a) and (b), respectively. The cells are nearly spherical before deformation as mentioned above. After compression, two cells are completely destroyed. Among the living cells, the left most one maintains round shape but the others show irregular morphology. This seems to be due to a local projection of cell membrane. It is interesting that even the cell with large projections is alive and maintains its membrane integrity. Dead or alive, the number of cells with irregular shape increased with decreasing gap size.

Figure 3 shows the effect of the gap size on the viability of deformed cells. The average value of the data obtained with each image is shown by a symbol with an error bar that expresses the standard deviation. The same

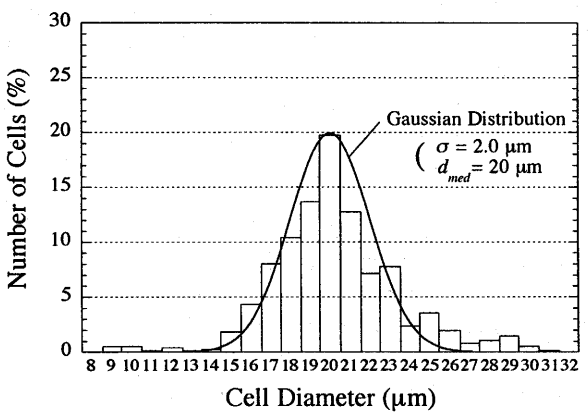
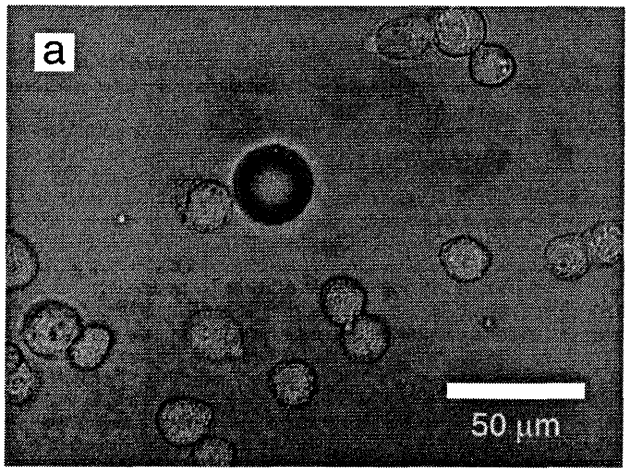
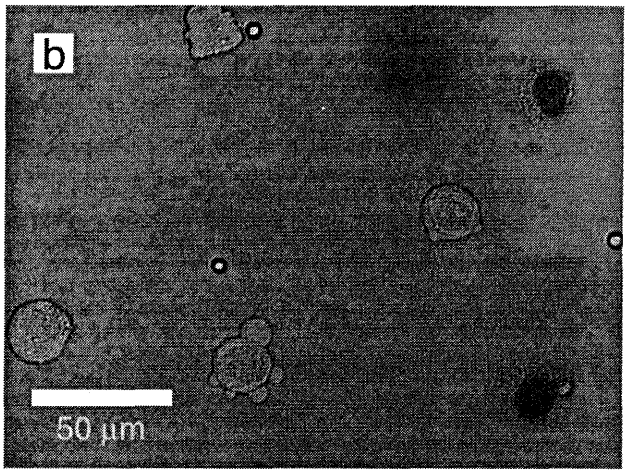


Fig. 1 Distribution of cell diameter.



(a) Control cells in a 32 μm gap.



(b) Deformed cells in a 5.9 μm gap.

Fig. 2 Close-up view of cells.

symbol denotes the same series of experiment. The symbols are shifted laterally to avoid overlap each other. Though a scatter is observed both in the mean value and the standard deviation, almost all the cells survive in the gap with h = 11.4 μm. The viability decreases slightly at h = 8.6 μm and significantly at h = 5.9 μm with decreasing gap size. This result demonstrates that about a half of cells are damaged at h = 5.9 μm that is 30% of original cell diameter.

A model for critical deformation

Here we assume an ideal deformation of a cell shown in Fig.4. The expansion rate γ of cell surface is evaluated using invariability of cell volume, and expressed as

$$\gamma = \frac{A}{A_0} = \left(-\frac{\pi^2}{16}+\frac{2}{3}\right)\left(\frac{h}{d_0}\right)^2 + \frac{1}{3}\left(\frac{d_0}{h}\right) + \frac{\pi}{4}\left(\frac{h}{d_0}\right)^2 \sqrt{\frac{\pi^2}{16}+\frac{2}{3}\left\{\left(\frac{d_0}{h}\right)^3 - 1\right\}}, \quad (2)$$

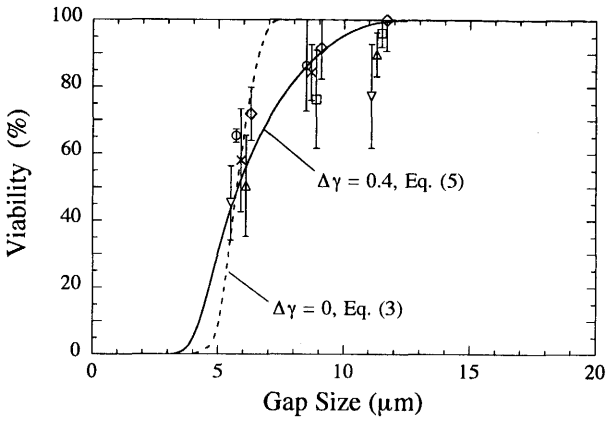


Fig. 3 Viability of deformed cells.

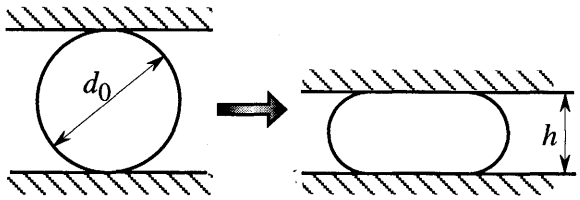


Fig. 4 Ideal deformation of a cell.

where A is the area of the cell surface and A_0 (= πd_0^2) is that of undeformed cell. Assuming uniform expansion of membrane and free slip at the plate-cell interface, we define a critical expansion rate γ_{cr}, that is the tolerable limit of expansion. The viability of individual cell η therefore comes

$$\eta(\gamma) = 1 \text{ for } \gamma < \gamma_{cr}, \quad (3a)$$

$$\eta(\gamma) = 0 \text{ for } \gamma \geq \gamma_{cr}. \quad (3b)$$

Since the cells used in the experiment have the size distribution expressed by Eq. (1), the viability obtained by the experiment is equivalent to the value obtained by the following equation:

$$\xi = \int_{d_{\min}}^{d_{\max}} f(d_0)\, \eta(\gamma)\, \mathrm{d}d_0. \quad (4)$$

Substituting Eqs. (1)-(3) for Eq. (4) and numerical integration yields the value of ξ for a given h. The dashed line in Fig. 3 denotes the calculated results with γ_{cr} = 1.5. It however shows an abrupt change which is much steeper than the experiment at $h \approx 6$ μm. The cells used in the experiment are at the various stage of growth cycle which seems to affect the mechanical property of the cell membrane. This is against the model that determines a unique value of γ_{cr}. Also, if compared with a tension test of industrial material, the limit of deformation corresponds to the fracture of material, which often depends on a few defects. Equation (3) is therefore replaced by the following equation to take into account of

variety of viability of individual cell:

$$\eta(\gamma) = 1 \text{ for } \gamma < \gamma_{cr} - \Delta\gamma, \quad (5a)$$

$$\eta(\gamma) = \frac{1}{2} - \frac{\gamma - \gamma_{cr}}{2\Delta\gamma} \text{ for } \gamma_{cr} - \Delta\gamma \le \gamma \le \gamma_{cr} + \Delta\gamma, \quad (5b)$$

$$\eta(\gamma) = 0 \text{ for } \gamma > \gamma_{cr} + \Delta\gamma. \quad (5c)$$

The solid line in Fig. 3 shows the results calculated using Eq. (5) with γ_{cr} = 1.5 and $\Delta\gamma$ = 0.4. This agrees well with the experimental data.

Summary

An experimental method was developed to evaluate the deformation limit of cells compressed by two parallel plates. With this method the viability of prostate cancer cells was obtained for different gap sizes at about 23 °C. The result demonstrated that the viability decreased significantly at the gap size about 30% of the original cell diameter, and a half of cells were damaged at this gap size. This result was preliminary expressed by a simple model which took into account of the critical expansion rate of the cell surface, variety of the critical value due to difference in cell growth cycle, and the distribution of the cell diameter.

The present method has the following advantages:

(1) The limit of deformation can be determined by the integrity of cell membrane. This is a necessary condition for the death of cell from the point of view of the cryosurgery.

(2) Membrane integrity is assessed at the situation that the cells are kept deformed.

(3) The viability is obtained statistically.

The problems are:

(1) Two plates must be parallel during deformation.

(2) The cells possibly undergo the shear stress during deformation, because suspension extends with decreasing gap size.

(3) The surface area that touches with the dye solution becomes small at a small gap.

In the present experiment, the cover slip was put on the suspension by hand, which sometimes caused the deviation of glass beads. Since this probably leads to the scattering of the data, a new method should be employed. Although evaluating the effect of the third problem quantitatively is impossible, the experimental result demonstrates that the dye exclusion assay seems to be possible even for a small gap size. It is obvious that much work remains to be done to correlate between cell compression and freezing damages. In particular, the effect of temperature on the limit of deformation should be examined because the cell membrane lipid has a phase transition temperature between 10 and 20 °C.

This research was conducted during the stay in University of California at Berkeley as the Visiting Scholar supported by the Ministry of Education, Japan. The author is grateful to Professor Boris Rubinsky of U. C. Berkeley for providing me an attractive topic on bioengineering.

References

1. Lovelock, J. E., The haemolysis of human red blood-cells by freezing and thawing, *Biochemica et Biophysica Acta*, **10** (1953), 414-426.
2. Lovelock J. E., The mechanism of protective action of glycerol against haemolysis by freezing and thawing, *Biochemica et Biophysica Acta*, **11** (1953), 28-36.
3. Pegg, D. E. and Diaper, M. P., On the mechanism of injury to slowly frozen erythrocytes, *Biophysical Journal*, **54** (1988), 471-488.
4. Pegg, D. E. and Diaper, M. P., The effect of initial tonicity on freeze/thaw injury to human red cells suspended in solutions of sodium chloride, *Cryobiology*, **28** (1991), 18-35.
5. Meryman, H. T., Modified model for the mechanism of freezing injury in erythrocytes, *Nature*, **218** (1968), 333-336.
6. Nei, T., Mechanism of hemolysis of erythrocytes by freezing at near-zero temperatures, II. Investigations of factors affecting hemolysis by freezing, *Cryobiology*, **4** (1968), 303-308.
7. Nei, T., Mechanism of freezing injury to erythrocytes: effect of initial cell concentration on the post-thaw hemolysis, *Cryobiology*, **18** (1981), 229-237.
8. Mazur, P., Rall, W. F. and Rigopoulos, N., Relative contributions of the fraction of unfrozen water and of salt concentration to the survival of slowly frozen human erythrocytes, *Biophysical Journal*, **36** (1981), 653-675.
9. Mazur, P. and Rigopoulos, N., Contributions of unfrozen fraction and of salt concentration to the survival of slowly frozen human erythrocytes: influence of warming rate, *Cryobiology*, **20** (1983), 274-289.
10. Ishiguro, H. and Rubinsky, B, Mechanical interactions between ice crystals and red blood cells during directional solidification, *Cryobiology*, **31** (1994), 483-500.
11. Evans, E. A. and Skalak, R., "*Mechanics and thermodynamics of biomembranes*," (1980) , CRC Press, Florida.
12. Bereiter-Hahn, J., Anderson, O. R. and Reif, W. -E. (Eds.), "*Cytomechanics*," (1987), Springer-Verlag, Berlin Heiderberg.
13. Mow, V. C., Guilak, F., Tran-Son-Tay, R. and Hochmuth, R. M. (Eds.), "*Cell Mechanics and Cellular Engineering*," (1994), Springer-Verlag, New York.
14. Onik, G. M. et al., "*Percutaneous prostate cryoablation*," (1995), Quality Medical Publishing, St. Louis.
15. Narayan, P. and Dahiya, R., Establishment and characterization of a human primary prostatic adenocarcinoma cell line (ND-1), *Journal of Urology*, **148** (1992), 1600-1604.
16 Hiramoto, Y., Mechanical properties of sea urchin eggs, *Experimental Cell Research*, **32** (1963), 59-75.